Vet Res Commun (2009) 33:811–821
DOI 10.1007/s11259-009-9229-0

ORIGINAL ARTICLE

Isolation, growth and differentiation of equine mesenchymal stem cells: effect of donor, source, amount of tissue and supplementation with basic fibroblast growth factor

Silvia Colleoni · Emanuela Bottani · Irene Tessaro · Gaetano Mari · Barbara Merlo · Noemi Romagnoli · Alessandro Spadari · Cesare Galli · Giovanna Lazzari

Accepted: 11 May 2009 / Published online: 27 May 2009

Abstract Mesenchymal stem cells (MSC) are increasingly used as therapeutical aid for the orthopaedic injuries in the horse. MSC populate different tissues but the most commonly used for clinical purposes are isolated from bone marrow or adipose tissue. The first objective of this study was to investigate if the donor animal, the tissue of origin and the technique of isolation could influence the number of MSC available for transplantation after a short-term expansion. The second aim was to devise a culture system capable of increasing MSC lifespan and we tested the effect of basic fibroblast growth factor (bFGF). Results indicate that MSC can be efficiently isolated from both sources and supplementation of bFGF enhances proliferation rate maintaining differentiation potential. In addition, this study shows that collection, expansion and storage of frozen MSC can be performed for later therapeutic use.

Keywords Mesenchymal stem cells · Equine · Orthopaedic injuries · Bone marrow · Adipose tissue

S. Colleoni (✉) · E. Bottani · I. Tessaro · C. Galli · G. Lazzari
Laboratorio di Tecnologie della Riproduzione, Avantea srl, Via Porcellasco 7/f, 26100 Cremona, Italy
e-mail: silviacolleoni@avantea.it

G. Mari · B. Merlo · N. Romagnoli · A. Spadari · C. Galli
Dipartimento Clinico Veterinario, Università di Bologna, Via Tolara di Sopra 50,
40064 Ozzano Emila, Italy

Present Address:
I. Tessaro
Sezione di Anatomia e Istologia Veterinaria, Dipartimento di Scienze Animali,
Facoltà di Medicina Veterinaria, Università degli Studi di Milano, Milano, Italy

Present Address:
E. Bottani
Istituto Nazionale Neurologico C.Besta, Milano, Italy

Introduction

The isolation of mesenchymal stem cells (MSC) has been described in several species and from different tissues, including bone marrow, peripheral blood, adult fat, umbilical cord blood and skeletal muscle. The main feature of MSC is the ability to differentiate both *in vitro* and *in vivo* along multiple pathways that include bone, cartilage, cardiac and skeletal muscle, tendon, connective and adipose tissue. (Pittenger et al. 1999). For this reason MSC-based cell therapies have been investigated for several years in human medicine and, more recently, the same approach has been considered in equine veterinary medicine as a novel potential therapy for horse musculoskeletal diseases (Frisbie 2005; Richardson et al. 2007; Smith et al. 2003).

In horses, mesenchymal stem cells from bone marrow have been isolated and characterized by several authors (Arnhold et al. 2007; Koerner et al. 2006; Vidal et al. 2006) and their multilineage differentiation potential has been demonstrated by the ability to undergo adipogenic, osteogenic and chondrogenic differentiation (Arnhold et al. 2007; Fortier et al. 1998; Vidal et al. 2006).

Fibroblast-like precursor cells have been also isolated from equine umbilical cord blood (Reed and Johnson 2008) and peripheral blood (Koerner et al. 2006), but in limited number and contrasting findings have been reported on their differentiation potential. More satisfactory results have been achieved with adult adipose tissue that has been proven a reliable source of MSC capable to differentiate into several mesenchymal derivatives in various species (Vidal et al. 2007; Zuk et al. 2002). Since these cells are readily accessible in large quantity in the horse, they have received increasing attention for their possible application for the treatment of musculoskeletal diseases. Recently, Vidal et al. have reported data on cell-doubling characteristics and differentiation ability of equine adipose-tissue derived stem cells in comparison to bone-marrow derived stem cells, showing that both cell types can be expanded and are able to differentiate into mesenchymal derivatives *in vitro*. Except for selected populations (Jiang et al. 2002), MSC are limited self-renewal stem cells. Basic fibroblast growth factor (bFGF) is often supplemented to culture media for several cell types to stimulate proliferation and for this reason it has been tested also on human bone marrow stromal cells. Results indicate that bFGF enhances growth potential during *in vitro* expansion (Bianchi et al. 2003) while maintaining their multidifferentiation ability (Stewart et al. 2007) (Solchaga et al. 2005; Sotiropoulou et al. 2006). Therefore the aim of this study has been to evaluate the proliferation potential of equine mesenchymal stem cells, derived from adipose tissue and from bone marrow and cultured in presence of basic fibroblast growth factor. In addition the data reported here provide quantitative practical information on the possibility of storing MSC for future use for the treatment of horse tendon injuries with autologous bone marrow or adipose tissue-derived mesenchymal stem cells.

Materials and methods

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich. Experimental procedures on animals were carried out in accordance with DL 116/92 and were approved by the Ministry of Health.

Bone marrow aspiration and isolation of bone marrow mesenchymal stem cells (BM-MSC)

Bone marrow was aspirated from the sternum and iliac crest of 4 horses, 2 to 4 years of age using 11 g bone marrow biopsy/aspiration needles. After multiple PBS (Dulbecco's

phosphate buffered solution) washings the cell suspension was loaded onto a 70% Percoll gradient and centrifuged at 1100 g for 30 min. Cells were collected from the interface and washed. Following counting the cells were plated at 2×10^5 cells/cm^2 and cultured in DMEM-TCM199 (1:1) + 10% FBS (Gibco, Invitrogen, Milan, Italy). Medium was replaced at 24 and 72 hours. After 1 week of culture the cell yield from different donor/sources was evaluated. Part of the cells from first passages was frozen.

Adipose tissue collection and isolation of adipose tissue stem cells (AT-MSC)

Adipose tissues were collected from the supragluteal subcuteneous area of 15 horses ranging from 2 to 9 years of age. Following several rinses in PBS the tissue was cut with a sterile scissor and incubated for 1 h at 38°C in hepes-buffered medium supplemented with 1 mg/ml collagenase (Collagenase Type IV, Gibco, Milan, Italy). The digested tissue was resuspended in serum containing medium and centrifuged. The remaining undigested tissue was incubated for an additional hour with collagenase and subsequently centrifuged. Pellets containing cells and tissue fragments were resuspended in culture medium (M199/DMEM 1:1 supplemented with 10% of FBS), washed once and plated in tissue culture 25 cm^2 flasks. Yield of cells after 1 week of culture was recorded. Part of the cells from the first passages was frozen.

Undifferentiated cell culture and population doublings

Sternal BM-MSC from donor n°13 and n°15 and AT-MSC from donor n°3 and n°15 were plated at a density of 5 X 10^3 cells/cm^2. Control cultures were maintained in medium TCM199/DMEM (1:1) supplemented with 10% fetal bovine serum (FBS), at 38.5°C in 5% O_2. Treated cultures were grown in the same conditions in presence of human bFGF (Peprotech, London, UK) at a final concentration of 5 ng/ml. Cells were cultured until they reached 80–90% confluence. Subsequently, cells were trypsinised, counted for the population doublings determination and reseeded at a density of 5×10^3 cells/cm^2. This procedure was repeated up to about 4 months of culture. Cell aliquots from different passages were used for *in vitro* differentiation as subsequently described.

Osteogenic differentiation

Equine AT-MSC and BM-MSC from different passages of the four lines used were plated at 3×10^3 cells/cm^2 in culture medium for 24 hours. Then the medium was replaced with osteogenic medium containing dexamethasone 100 nM, ascorbic acid 0.25 mM and β-glycerolphosphate 10 mM. After 8–12 days of culture, the cells were stained to reveal alkaline phosphatase activity (BCIP/NBT, Lab Vision Corp., Fremont, CA). Calcium accumulation was detected by Von Kossa staining after 21 days of culture. Alkaline Phosphatase and Von Kossa staining were performed on undifferentiated AT-MSC and BM-MSC cultured in non-inductive medium to detect the endogenous activity. Additionally, equine adult fibroblasts were cultured in the same culture conditions as negative controls.

Chondrogenic differentiation

Chondrogenic differentiation was performed in pellet culture on different passages of equine AT-MSC and BM-MSC from donor n°15. Approximately 5×10^5 cells were suspended in 2 ml of chondrogenic medium, containing dexamethasone 100 nM, ascorbic

acid 0.25 mM, 5 ng/ml of TGFβ1 (Peprotech, London, UK) and ITS (ITS premix, VWR International, Milan, Italy) placed in a 10 ml-tube, centrifuged at 500 rpm for 7 minutes and incubated at 38.5°C in 5% O_2. After 3 weeks, cells were stained with Alcian Blue to detect glycosamminoglicans content.

Occasionally chondrogenic differentiation was performed in monolayer under the same culture conditions. Alcian Blue staining was performed also on AT-MSC and BM-MSC cultured in non-inductive medium to detect a basal signal. Additionally, equine adult fibroblasts were cultured in the same conditions as a negative control.

RNA extraction and semi-quantitative gene expression analysis

Total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) from samples of undifferentiated and differentiated cells. The RT reaction was performed using a First strand cDNA synthesis kit (Fermentas, Ontario, Canada) according to the instructions of the manufacturer. PCR reaction was performed using an MJ Minicycler and Hot StarTaq Polymerase (Qiagen, Hilden, Germany). After RT-PCR reaction electrophoresis was performed on a 2.5% agarose gel. The results of the electrophoresis were captured with a Gel Logic 100 Kodak Imaging System and images were analysed with a Kodak Molecular Imaging Software. The intensity of each band was analysed by densitometry and the results were normalised using 18 S as external standard. The following primers were used (Giovannini et al. 2008): 18 S: FW 5′CGGCTACCACATCCAAGGAA 3′, RV 5′ GCTGGAATTACCGCGGCT 3′ ; Aggrecan: FW 5′ ACAACAATGCCCAAGACTAC 3′, RV 5′ GCCAGTTGTCAAATTGCAAG 3′; Collagen Type II: FW 5′ TGAAACTCTGC CACCCTGAATG 3′, RV 5′ TTGTCCTTGCTCTTGCTGCTC 3′; Osteonectin: FW 5′ AGACCTTCGATTCCTCCTGC 3′, RV 5′ TTGCCCTCATCCCTTTCG 3′. Osteonectin sequence was identified in equus caballus genome (XM001503651) by homology (93.8%) with human sequence (NM_003118). Primers for osteonectin were designed using the software VectorNTI 10 Advance and PCR product was sequenced obtaining an identity score of 94.6%.

Statistical analysis

Statistical analysis was done performing Student T test. Values were considered statistically different when P was<0.05.

Results

Isolation and expansion of equine AT-MSC and BM-MSC and cell doubling data

Cell yields obtained after 1 week of culture from different donors and sources are listed in Tables 1 and 2.

In Table 1 are shown the data referring to the efficiency of isolation of MSC from adipose tissue from 11 horses and the corresponding cell yield obtained after one week from the initial plating. Similarly in Table 2 are shown the data referring to MSC collection from 4 additional donors and from different sources: adipose tissue, sternum and iliac crest. The data reported in Table 1 indicate that after one week of expansion the yield of MSC varies from 5×10^5 to 5×10^6 and this number is not always related to the amount of starting tissue: for instance 12 g of adipose tissue from donor 4 provided 5×10^5 cells after one week

Table 1 Isolation of MSC from adipose tissue of different donors

Donor	Age	Quantity of tissue (gr)	Yield after 1 week	Cells/gr
1	4	11	4000000	363636
2	8	5	800000	160000
3	2	11	1600000	145455
4	9	12	500000	41667
5	4	12	2000000	166667
6	4	19	4000000	210526
7	4	19	4000000	210526
8	2	10	2800000	280000
9	4	19	5200000	273684
10	2	22	5000000	227273
11	2	18	4000000	222222

while 11 g from donor 1 provided 4×10^6 cells in the same culture time. Comparing the data reported in Table 1 with those of Table 2 it is clear that the amount of starting tissue is much higher in the second group of animals most likely due to the different operators who performed the sampling. Interestingly, the number of cells per gram of tissue obtained after one-week expansion from group 1 (average 209,000 cells/gram) is significantly higher (T Student test. $P<0.05$) than the average obtained from group 2 (108,000 cells/gram). This indicates that the digestion of the adipose tissue has been relatively inefficient from the second group of animals suggesting that the digestion procedure must be adapted (higher enzyme concentration/longer exposure) to the amount of tissue to be processed in order to optimise results, also if we cannot exclude an intrinsic variability between samples. We also found a significant (T test, $p=0.03$) lower yield of cells for gram of tissue from the samples of the two older horses (donor number 2 and 4), however due to the low number of horses in this group data need to be further confirmed.

All cultured cells showed fibroblastic morphology (Fig. 1a) and the supplementation of bFGF induced a slight change towards a more spindle shaped morphology (Fig. 1b).

Table 2 Isolation and yield of MSC from different sources

Donor	Age	Type of tissue	Quantity of tissue	Yield after 1 week	Cells/Tissue
12	2	Adipose tissue	50 gr	6000000	120000
		Sternal bone marrow	100 ml	8000000	80000
		Iliac crest bone marrow	50 ml	3000000	60000
13	2	Adipose tissue	40 gr	5000000	125000
		Sternal bone marrow	50 ml	6000000	120000
		Iliac crest bone marrow	80 ml	5000000	625000
14	2	Adipose tissue	57 gr	5000000	87719
		Sternal bone marrow	55 ml	6000000	109090
		Iliac crest bone marrow	75 ml	6000000	80000
15	4	Adipose tissue	61 gr	6000000	98360
		Sternal bone marrow	120 ml	6000000	50000
		Iliac crest bone marrow	75 ml	5000000	66666

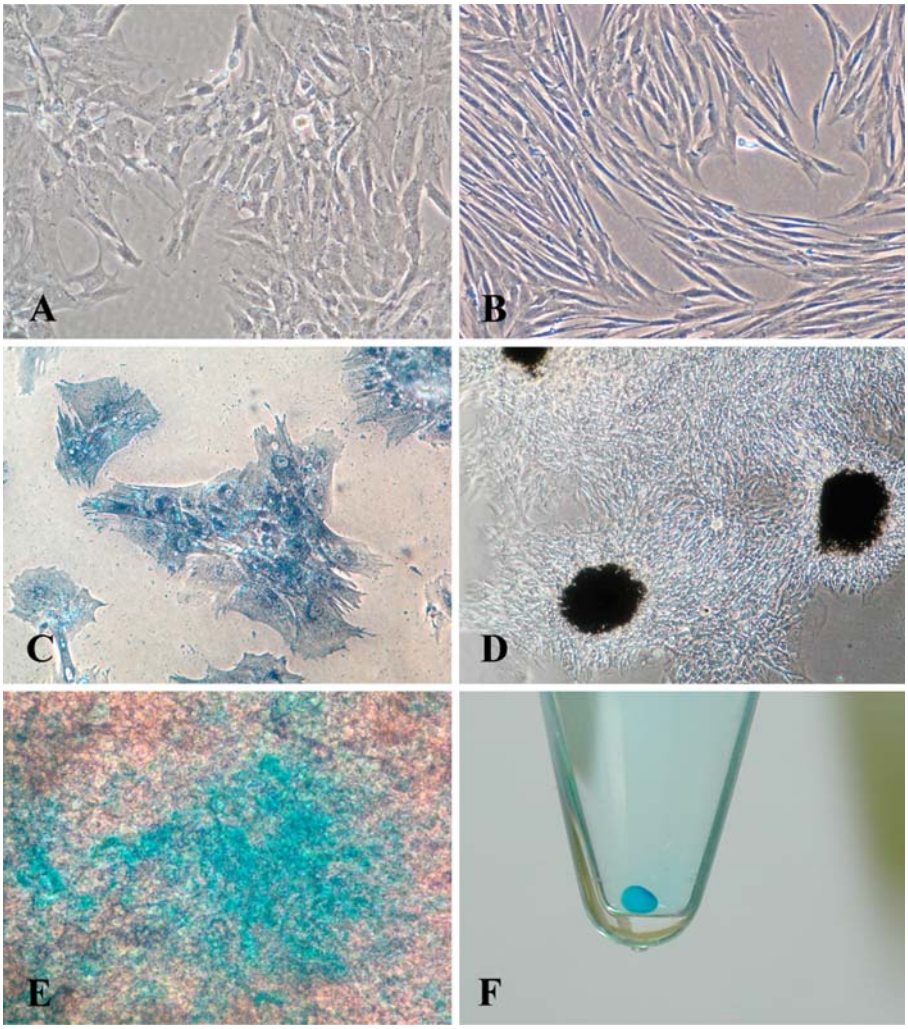

Fig. 1 a-b: Undifferentiated adipose tissue-derived mesenchymal stem cells untreated (a) and treated with bFGF (b). c-d: Osteogenic differentiation: alkaline phosphatase detection after 10 days in osteogenic medium (c) and von kossa staining after 21 days of culture (d). e-f: Chondrogenic differentiation: alcian blue staining of cells treated for 21 days with chondrogenic medium in monolayer (e) or in pellet (f)

Growth characteristics of equine BM-MSC and AT-MSC are summarized in Table 3 and Fig. 2. The data indicate, under our experimental conditions, a higher proliferation potential of AT-MSC as compared to BM-MSC, proliferation that was further enhanced by bFGF supplementation.

In this study we have cultured 2 lines of equine AT-MSC and 2 lines of equine AT-MSC up to 90-120 days to evaluate proliferation rate and doubling time (see Table 3). Our data indicate that the AT-MSC lines have a higher proliferative ability (population doublings) as compared to the BM-MSC lines although not statistically different (T test, $p=0.12$). Moreover, we found that the supplementation of bFGF considerably increased proliferation rate of both BM-MSC and AT-MSC lines with statistically significant effect for AT-MSC (T

Table 3 Cell growth characteristics of equine AT and BM-MSC cultured in control conditions and treated with 5 ng/ml of bFGF

Cell type	Culture days	Population doublings (Doubling time) *	Yield after 3 weeks from isolation	Yield after 6 weeks from isolation
AT-MSC				
Donor 3	92	36.5 (2.5)	144×10^{6}	45.6×10^{9}
Donor 15	122	28.9 (4.2)	348×10^{6}	10.7×10^{9}
AT-MSC(+)FGF**				
Donor 3	95	67.7 (1.4)	6.5×10^{9}	17×10^{14}
Donor 15	112	64.6 (1.7)	393×10^{9}	415×10^{14}
BM-MSC				
Donor 13	100	13.3 (7.5)	89×10^{6}	2.7×10^{9}
Donor 15	118	22.1 (5.3)	34×10^{6}	623×10^{6}
BM-MSC(+)FGF**				
Donor 13	85	32.6 (2.6)	2.9×10^{9}	25×10^{12}
Donor 15	112	37.9 (2.9)	11.5×10^{9}	25×10^{12}

*Population doublings and doubling time are calculated starting from week 1 after isolation

** FGF treatment has been started 1 week after isolation. See Table 1

test, p=0.015) and value near the significant level for BM-MSC (T test, p=0.075). In order to provide an estimate of the number of cells that can be obtained from the different sources with or without bFGF supplementation during culture, we have calculated (Table 3) the cell yield after three and six weeks expansion. Interestingly, referring to donor 15, the data on cell number after one week of culture (Table 2) indicate an equal number of cells obtained from adipose tissue and from sternal bone marrow. However, the cell yield after three weeks is over ten times higher for AT-MSC than for BM-MSC. The supplementation with bFGF amplifies this difference by a factor of three and comparing mean proliferation ability for the two donors in presence of FGF the difference between sources was statistically significant (T test, p=0.01). Considering the cell yield after six weeks of culture both the source of cells and the supplementation of bFGF further increase the differences observed above. Similar data have been obtained with AT-MSC derived from donor 3 and BM-MSC obtained from donor 13.

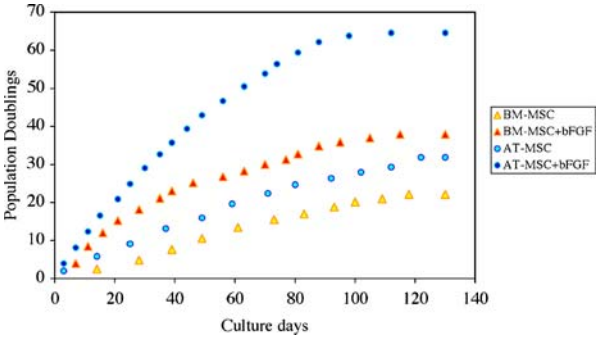

Fig. 2 Representative growth curves of the lines derived from adipose tissue and bone marrow of donor n°15 grown in control medium or in presence of bFGF

Culture differentiation

Following induction of osteogenic differentiation we observed a clear change of morphology in all cell lines tested. Cells acquired a polygonal shape within three days and cell-aggregates and nodule formation were observed after 4–6 days of culture. Alkaline phosphatase activity (Fig. 1c) was increased in comparison to endogenous basal activity and matrix mineralization and calcium accumulation were detected by Von Kossa staining after 21 days of culture (Fig. 1d).

Both AT-MSC and BM-MSC cultured with bFGF supplementation displayed similar morphological changes after exposure to osteogenic medium and qualitative macroscopical observation performed by different operators showed that alkaline phosphatase activity was increased in comparison to control cultures. As previously observed, calcification appeared as several black regions within the cell monolayer. When the differentiation potential of equine mesenchymal stem cells was compared to equine adult fibroblasts, we found that fibroblast cells did not respond to osteogenic induction. Only sporadic cells were positive for alkaline phosphatase, whereas calcium accumulation was undetectable, suggesting an undifferentiated phenotype, although the cells did not appear healthy and extensive cell death was observed in these culture conditions.

All cell lines underwent chondrogenic differentiation at much higher level in pellet culture (Fig. 1f) as compared to monolayer culture (Fig. 1e), with no significant decrease with increasing population doublings.

The expression data from RT-PCR analysis are illustrated in Fig. 3 and indicated the presence of a basal expression of osteonectin and aggrecan in undifferentiated MSC that increased after treatment with osteogenic and condrogenic medium respectively. By contrast, the condrogenic marker collagen II was expressed in chondro-differentiated cultures while was undetected in undifferentiated MSC. Treatment with FGF increased the expression of osteonectin both in undifferentiated and differentiated cells but not at statistically significant level (T test, $p=0.2$ and $p=0.09$ for undifferentiated and differentiated cells respectively)

Discussion

In this study, we compared equine MSC from bone marrow and adipose tissue for growth rate, differentiation ability and influence of bFGF on these features. We observed differences in cells yield from different donors especially following isolation from adipose tissue. This finding indicates not only donor specific differences but also that digestion procedure must be adapted to the amount of tissue as we found in more recent experiments (personal observation). We evaluated 4 cell lines of equine BM-MSC and AT-MSC, and we found relevant differences in cell growth characteristics. All the MSC lines maintained their differentiation ability throughout the culture period although a progressive reduction in the efficiency of differentiation was observed in the last passages. This reduction is likely the consequence of cell senescence that was morphologically evident also in the late passages of the undifferentiated cultures. Similar differences in growth potential of mesenchymal stem cells are reported for human MSC: while some cell lines can be expanded for more than 15 cell doublings, for others proliferation is limited to 4 cell doublings (Bruder et al. 1997; Digirolamo et al. 1999). Critical parameters for MSC isolation include donor variability, tissue of origin, cell selection and enrichment, culture surface coating, culture media, oxygen tension, serum supplements, cell density of culture, which all can have impact on morphology and composition of cell preparations (Sotiropoulou et al. 2006; Wagner and Ho 2007). Based on our results, mesenchymal cells

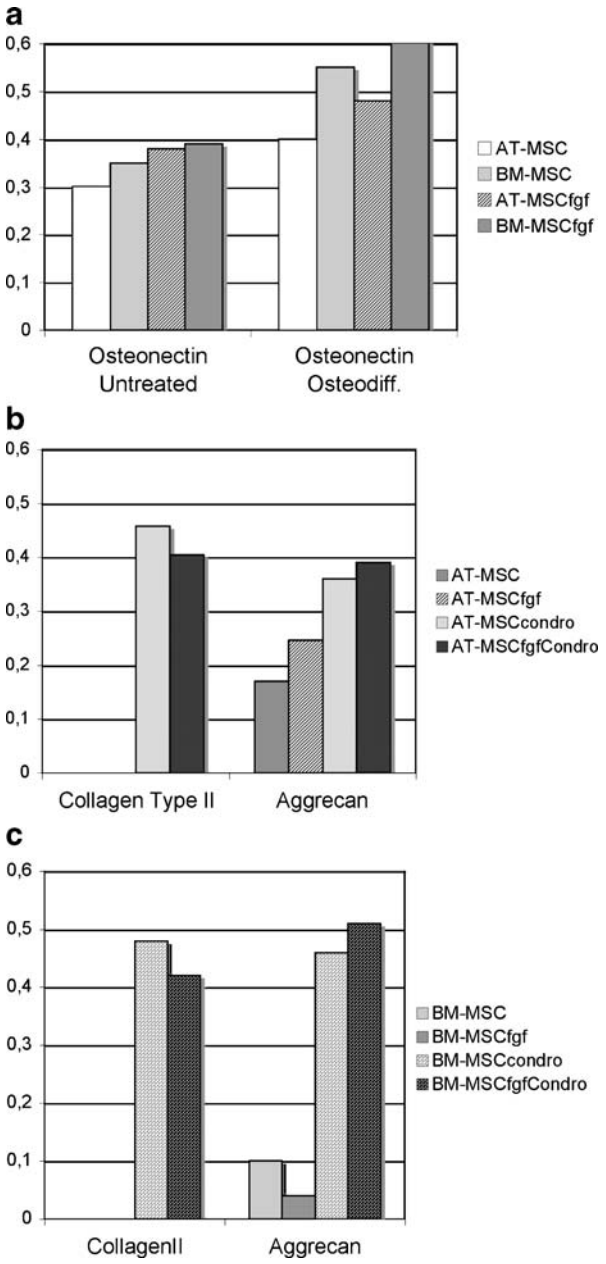

Fig. 3 a: Relative expression of osteonectin in AT-MSC(+/-fgf) and BM-MSC (+/-fgf) undifferentiated and differentiated for 1 week in osteogenic medium. b-c: Relative expression of collagen type II and Aggrecan in AT-MSC (+/-fgf) (b) and BM-MSC (+/-fgf) (c) differentiated in condrogenic medium for 10 days

from adipose tissue are superior to BM-MSC regarding proliferating ability both for total number and doubling time, even considering that the starting number of AT-MSC was lower than BM-MSC. Similar finding have been previously described (Izadpanah et al. 2006; Kern et al. 2006; Lee et al. 2004) although other authors did not find any significant differences among MSC from different sources (De Ugarte et al. 2003).

To test the effect of bFGF on equine MSC, we compared growth rate and population doublings of cells cultured in control medium and in medium supplemented with bFGF. We

found that the addition of bFGF increased growth rate and population doublings of equine MSC irrespective of tissue of origin as previously found in other species (Bianchi et al. 2003; Ito et al. 2007; Solchaga et al. 2005; Sotiropoulou et al. 2006; van den Bos et al. 1997). Moreover, in agreement with our findings, previous reports on various species showed that bFGF treatment could increase differentiation ability of MSC *in vitro* (Stewart et al. 2007) (Hanada et al. 1997; Sotiropoulou et al. 2006) and bone synthesis in *in vivo* models by stimulating osteoprogenitor cells (Amizuka et al. 1998). We also found that bFGF treatment had a tendency, also if not statistically significant, to increase osteonectin mRNA expression level both in AT and BM-MSC when cultured in osteogenic induction medium.

Finally, the long-term cultures performed in this study demonstrated that MSC from all the three donors examined and especially if supplemented with bFGF, represent a large reservoir of cells for potential clinical use. In this study we consider for long-term analysis 4 out of 23 lines derived and also if large variability between donors was observed, we were able to conclude that in the context examined, the collection, expansion and storage of frozen MSC, can be proposed as a preventive strategy for those valuable sporting horses that are most exposed to the risk of ligament and skeletal injuries. In this way autologous MSC, available in large quantities, just after thawing and short overnight culture, could represent a ready-to-use therapeutic option/aid for orthopaedic diseases. In conclusion, we demonstrated, although in a limited number of donors, that equine AT-MSC are a better source of progenitor cells regarding growth rate and population doubling number than BM-MSC, and have a greater response to the addition of bFGF in culture medium. Moreover, we showed that after bFGF treatment, MSC retain the ability to differentiate into osteogenic and chondrogenic lineages, a finding that could have important implications in veterinary tissue engineering and regenerative therapies. Controlled clinical studies are the next step required to fully evaluate the effects of bFGF supplementation during culture on the therapeutic potential of MSC for the treatment of equine orthopaedic injuries.

Acknowledgments We thank Dr. Andrea Perota for osteonectin primers design and analysis of sequence homology.

Funding This work was supported by grants from MUR (PRIN and TECLA), Fondazione Cariplo (NOBEL)

References

Amizuka N, Yamada M, Watanabe JI, Hoshi K, Fukushi M, Oda K, Ikehara Y, Ozawa H (1998) Morphological examination of bone synthesis via direct administration of basic fibroblast growth factor into rat bone marrow. *Microsc Res Tech* **41**, 313–22. doi:10.1002/(SICI)1097-0029(19980515)41:4<313::AID-JEMT4>3.0.CO;2-R

Arnhold SJ, Goletz I, Klein H, Stumpf G, Beluche LA, Rohde C, Addicks K, Litzke LF (2007) Isolation and characterization of bone marrow-derived equine mesenchymal stem cells. *Am J Vet Res* **68**, 1095–105. doi:10.2460/ajvr.68.10.1095

Bianchi G, Banfi A, Mastrogiacomo M, Notaro R, Luzzatto L, Cancedda R, Quarto R (2003) *Ex vivo* enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. *Exp Cell Res* **287**, 98–105. doi:10.1016/S0014-4827(03)00138-1

Bruder SP, Jaiswal N, Haynesworth SE (1997) Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J Cell Biochem* **64**, 278–94. doi:10.1002/(SICI)1097-4644(199702)64:2<278::AID-JCB11>3.0.CO;2-F

De Ugarte DA, Morizono K, *et al.* (2003) Comparison of multi-lineage cells from human adipose tissue and bone marrow. *Cells Tissues Organs* **174**, 101–9. doi:10.1159/000071150

Digirolamo CM, Stokes D, Colter D, Phinney DG, Class R, Prockop DJ (1999) Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the

greatest potential to propagate and differentiate. *Br J Haematol* **107**, 275–81. doi:10.1046/j.1365-2141.1999.01715.x

Fortier LA, Nixon AJ, Williams J, Cable CS (1998) Isolation and chondrocytic differentiation of equine bone marrow-derived mesenchymal stem cells. *Am J Vet Res* **59**, 1182–7.

Frisbie DD (2005) Future directions in treatment of joint disease in horses. *Vet Clin North Am Equine Pract* **21**, 713–24, viii. doi:10.1016/j.cveq.2005.07.001

Giovannini S, Brehm W, Mainil-Varlet P, Nesic D (2008) Multilineage differentiation potential of equine blood-derived fibroblast-like cells. *Differentiation* **76**, 118–29. doi:10.1111/j.1432-0436.2007.00207.x

Hanada K, Dennis JE, Caplan AI (1997) Stimulatory effects of basic fibroblast growth factor and bone morphogenetic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. *J Bone Miner Res* **12**, 1606–14. doi:10.1359/jbmr.1997.12.10.1606

Ito T, Sawada R, Fujiwara Y, Seyama Y, Tsuchiya T (2007) FGF-2 suppresses cellular senescence of human mesenchymal stem cells by down-regulation of TGF-beta2. *Biochem Biophys Res Commun* **359**, 108–14. doi:10.1016/j.bbrc.2007.05.067

Izadpanah R, Trygg C, Patel B, Kriedt C, Dufour J, Gimble JM, Bunnell BA (2006) Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. *J Cell Biochem* **99**, 1285–97. doi:10.1002/jcb.20904

Jiang Y, Jahagirdar BN, *et al.* (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**, 41–9. doi:10.1038/nature00870

Kern S, Eichler H, Stoeve J, Kluter H, Bieback K (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* **24**, 1294-301. doi:10.1634/stemcells.2005-0342

Koerner J, Nesic D, Romero JD, Brehm W, Mainil-Varlet P, Grogan SP (2006) Equine peripheral blood-derived progenitors in comparison to bone marrow-derived mesenchymal stem cells. *Stem Cells* **24**, 1613–9. doi:10.1634/stemcells.2005-0264

Lee RH, Kim B, Choi I, Kim H, Choi HS, Suh K, Bae YC, Jung JS (2004) Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. *Cell Physiol Biochem* **14**, 311–24. doi:10.1159/000080341

Pittenger MF, Mackay AM, *et al.* (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–7. doi:10.1126/science.284.5411.143

Reed SA, Johnson SE (2008) Equine umbilical cord blood contains a population of stem cells that express Oct4 and differentiate into mesodermal and endodermal cell types. *J Cell Physiol* **215**, 329–36. doi:10.1002/jcp.21312

Richardson LE, Dudhia J, Clegg PD, Smith R (2007) Stem cells in veterinary medicine–attempts at regenerating equine tendon after injury. *Trends Biotechnol* **25**, 409–16. doi:10.1016/j.tibtech.2007.07.009

Smith RK, Korda M, Blunn GW, Goodship AE (2003) Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment. *Equine Vet J* **35**, 99–102. doi:10.2746/042516403775467388

Solchaga LA, Penick K, Porter JD, Goldberg VM, Caplan AI, Welter JF (2005) FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. *J Cell Physiol* **203**, 398–409. doi:10.1002/jcp.20238

Sotiropoulou PA, Perez SA, Salagianni M, Baxevanis CN, Papamichail M (2006) Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. *Stem Cells* **24**, 462–71. doi:10.1634/stemcells.2004-0331

Stewart AA, Byron CR, Pondenis H, Stewart MC (2007) Effect of fibroblast growth factor-2 on equine mesenchymal stem cell monolayer expansion and chondrogenesis. *Am J Vet Res* **68**, 941–5. doi:10.2460/ajvr.68.9.941

van den Bos C, Mosca JD, Winkles J, Kerrigan L, Burgess WH, Marshak DR (1997) Human mesenchymal stem cells respond to fibroblast growth factors. *Hum Cell* **10**, 45–50.

Vidal MA, Kilroy GE, Johnson JR, Lopez MJ, Moore RM, Gimble JM (2006) Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity. *Vet Surg* **35**, 601–10. doi:10.1111/j.1532-950X.2006.00197.x

Vidal MA, Kilroy GE, Lopez MJ, Johnson JR, Moore RM, Gimble JM (2007) Characterization of equine adipose tissue-derived stromal cells: adipogenic and osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells. *Vet Surg* **36**, 613–22. doi:10.1111/j.1532-950X.2007.00313.x

Wagner W, Ho AD (2007) Mesenchymal stem cell preparations–comparing apples and oranges. *Stem Cell Rev* **3**, 239–48. doi:10.1007/s12015-007-9001-1

Zuk PA, Zhu M, *et al.* (2002) Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* **13**, 4279–95. doi:10.1091/mbc.E02-02-0105